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<b>(21) International Application Number:</b> PCT/CA98/00581  <b>(22) International Filing Date:</b> 12 June 1998 (12.06.98)  <b>(30) Priority Data:</b> 08/874,663                      13 June 1997 (13.06.97)                      US  <b>(71) Applicant:</b> ALLELIX BIOPHARMACEUTICALS INC. [CA/CA]; 6850 Goreway Drive, Mississauga, Ontario L4V 1V7 (CA).  <b>(72) Inventors:</b> BALLYK, Barbara, Ann; 2 Briarcroft Road, Toronto, Ontario M6S 1H4 (CA). ZASTAWNY, Roman; 21 Newstead Road, Etobicoke, Ontario M9P 3G2 (CA). LEE, David, K., H.; 5309 Forest Hill Drive, Mississauga, Ontario L5M 5B8 (CA). DEMCHYSHYN, Lidia; 4 Fernalroy Boulevard, Etobicoke, Ontario M8Z 3V6 (CA). CATALANO, Concettina; 119 Claremont Lane, Woodbridge, Ontario L4L 8Z8 (CA).  <b>(74) Agents:</b> CHARI, Santosh, K. et al.; Orange & Associates, Toronto Dominion Bank Tower, Suite 3600, Toronto-Dominion Centre, P.O. Box 190, Toronto, Ontario M5K 1H6 (CA).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> ASSAY METHODS AND COMPOSITIONS USEFUL FOR MEASURING RECEPTOR LIGAND BINDING  <b>(57) Abstract</b>  <p>This invention provides a system for screening chemical compounds to identify ligands for receptors including G-protein coupled receptors. The invention exploits cells in which the receptor is coupled through a second messenger system to an ion channel that is gated by cyclic nucleotide. Receptor stimulation causes the second messenger system to produce cyclic nucleotide, which results in ion influx through the channel. By measuring ion influx fluorescently, the invention provides a rapid and convenient means for identifying receptor ligands. By providing mixed cell cultures that include cells expressing different receptor types, and by loading into those cells different fluorescent reporters of ion influx, the invention further provides a multiplexed system that accelerates the ligand identification process. Cells useful in the process, and methods for exploiting them, are described.</p>		

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## ASSAY METHODS AND COMPOSITIONS USEFUL FOR MEASURING RECEPTOR LIGAND BINDING

### 5 Field of the Invention

This invention is in the field of molecular biology, particularly as applied to pharmaceuticals. More particularly, the invention relates to methods and discovery. compositions for detecting receptor ligand binding and to their use in drug

### 10 Background of the Invention

A wide variety of cell surface receptors have been implicated in disease processes, and are accordingly targets of drug discovery programs, which seek to identify ligands for these receptors. To facilitate the discovery of such ligands, cells that express these receptors and report the presence of bound ligands have been developed; for instance, assays which assess ligand  
15 binding by monitoring alteration in the level of a second messenger, such as cAMP, cGMP or inositoltriphosphate (IP<sub>3</sub>) are established and have been automated, allowing them to be used in high-throughput and ultra high-throughput screening of chemical libraries for ligands. In one approach, the effect of ligand binding at the receptor is revealed by a reporter gene product whose expression is driven by second messengers stimulated upon ligand binding at  
20 the receptor. Typically, the reporter gene codes for a readily detectable protein product, for example, CAT or luciferase (see for example US 5,436,128 and US 5,401,629). A related system has been developed in which ligand binding at a target receptor is reported by the formation of a pigment protein (see US 5,462,856). Although expression of a reporter gene can be stimulated rapidly in systems of this type, detection of that response is delayed until  
25 the expression product of the reporter gene is formed.

It is an object of the present invention to provide an alternative system to identify receptor ligands rapidly and efficiently.

### 30 Summary of the Invention

To assess the receptor binding properties of chemical compounds, there is provided by the present invention a cell-based system in which binding of a ligand to the receptor target is reported rapidly and conveniently by assessing ion flux across the cell membrane. This is achieved using cells that produce both the target receptor and an ion channel protein, as well

as a second messenger system that allows the flow of ions through the channel protein to be gated in response to a ligand binding event at the target receptor. Detection of altered ion channel activity, particularly altered ion flow, thereby reports the presence of a target receptor ligand, in the rapid manner useful for accelerated drug discovery.

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In one aspect, the invention therefore provides a method for identifying receptor ligands, which comprises the steps of obtaining a cell useful to screen for receptor ligands, the cell expressing a receptor target and an ion channel wherein gating of the ion channel is influenced by the level of a second messenger, incubating a candidate ligand under receptor binding conditions, such that binding of a ligand to the receptor results in an alteration in that second messenger, and then determining if a change in the activity of the ion channel has occurred. In embodiments of the invention, the method is adapted to allow for the identification of ligands that are agonists at the receptor target, and ligands that are antagonists at the receptor target.

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In a related aspect, the invention provides a cell that is genetically adapted to produce a receptor target and an ion channel, at least one and preferably both of which is encoded by a heterologous nucleic acid, wherein binding of a ligand to the receptor protein alters the intracellular concentration of a secondary messenger, this alteration modulating ion flow across the ion channel when a ligand is bound by the receptor target.

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In preferred aspects, the ion channel is a cyclic nucleotide gated channel. In further preferred aspects, the receptor target is a G-protein coupled receptor target.

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In a related further aspect, the invention provides a method for screening chemical compounds in a multiplexed fashion to identify G-protein coupled receptor ligands, comprising the steps of:

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- (a) obtaining a mixed culture of cells in which each cell is adapted genetically to produce (i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target; wherein said mixed culture of cells includes a first cell type that produces a first type of G-protein coupled receptor and a second cell

type that produces a second type of G-protein coupled receptor different from said first type of G-protein coupled receptor;

(b) incubating the mixed culture of cells with at least one G-protein coupled receptor ligand candidate; and

(c) determining the effect of the receptor ligand candidate on ion channel activity, preferably by determining ion flux through the channel.

10 In a further aspect, the present invention provides, for use in the multiplexed method of the present invention, a mixed culture of cells in which each cell is adapted genetically to produce (i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target; wherein said mixed culture of cells includes a first cell type as defined above, and a second cell type that produces a species of G-protein coupled receptor different from the G-protein coupled receptor species produced by the first cell type.

20 Embodiments of the invention are described in greater detail with reference to the accompanying drawings in which:

Brief Reference to the Drawings:

25 Figure 1 illustrates the fluorescence response of CNG channel-producing cells (filled shapes) and control cells (open shapes) to incubation with either a cyclic nucleotide (8Br-cGMP) or forskolin;

Figure 2 illustrates the effect on calcium influx of increasing concentrations of the cyclic nucleotides 8Br-cGMP (panel A) and 8Br-cAMP (panel B) as well as forskolin (panel C) in CNG channel producing cells of Figure 1;

30 Figure 3 illustrates the specific fluorescence response of cells stably producing both CNG channel and 5HT6 receptor, to 5HT6 receptor selective agonists (5-HT, 5-CT and 5-MeOT, panel A) and to 5HT6 receptor selective antagonists (clozapine and methiothepin, panel B);

Figure 4 illustrates the specific fluorescence response of cells stably producing both CNG channel and D1 receptor, to D1 receptor agonists (dopamine, ADTN, and SKF38393, panel A) and to D1 receptor antagonists (flupentixol and SCH23390, panel B);

- 5 Figure 5 illustrates specific fluorescence response of cells stably producing both CNG channel and D1 receptor, to D1 receptor agonists as a control (panel A) and to D1 receptor agonists when incubated 5 minutes after treatment with D1 receptor antagonist (panel B);

- 10 Figure 6 illustrates the specific fluorescence responses of a mixed culture containing two cell lines producing either CNG channel loaded with Fura-Red (panels A and B) or CNG channel and D1 receptor loaded with Fluo-3 (panels C and D) Response of cells loaded with Fluo-3 was measured as an increase in fluorescence, while response of cells loaded with Fura Red was measured as a decrease in fluorescence;

- 15 Figure 7 illustrates the specific fluorescence responses to reference compounds, including D1 and 5HT6 receptor reference agonists, of a mixed culture containing two cell lines producing either CNG channel and 5HT6 receptor loaded with Fura Red, or CNG channel and D1 receptor loaded with Fluo-3; and

- 20 Figure 8 illustrates the identification of D1 and 5HT6 reference antagonists in a mixed culture containing two cell lines producing either CNG channel and 5HT6 receptor or CNG channel and D1 receptor loaded with Fura Red and Fluo-3 respectively.

#### Detailed Description of the Invention

- 25 According to the present invention, there are created and exploited cells that produce both a receptor target and an ion channel, and which further have a second messenger system through which binding activity at the receptor target results in detectable activity at the ion channel. The invention is applicable to cells expressing a wide variety of receptor targets, and a wide variety of ion channels.

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The term "receptor target" is used herein with reference to protein molecules that occur on the surface of cells which interact with the extracellular environment, and transmit or transduce that external information in a manner that ultimately modulates the intracellular environment.

Cell surface-localized receptors are membrane spanning proteins that bind extracellular signalling molecules and transmit the signal via signal transduction pathways to effect a cellular response. Cell surface receptors bind circulating signal molecules, such as growth factors and hormones, as the initiating step in the induction of numerous second messenger pathways. Receptors are classified on the basis of the particular type of pathway that is induced. Included among these classes of receptors are those that bind growth factors and have intrinsic tyrosine kinase activity, such as the heparin binding growth factor (HBGF) receptors, and those that couple to effector proteins through guanine nucleotide binding regulatory proteins, which are referred to as G protein coupled receptors (GPCR) and G proteins, respectively.

The term "ion channel" refers to membrane spanning proteins that permit controlled entry of various ions into cells from the extracellular fluid. They function as gated pores in the cell membrane and permit the flow of ions down electrical or chemical gradients. Ion channels are classified on the basis of the ion that enters the cell via the channel. The modulation of transmembrane ion transport is often the primary event in the coupling of extracellular signals to intracellular events. Ion fluxes play essential roles in stimulus-mitosis, stimulus-contraction (see, Curran et al. (1986) Proc Natl. Acad. Sci. USA 83:8521-8524). For example, the voltage-gating of calcium ions mediates the coupling of membrane depolarizing stimuli to transcriptional activation of c-fos gene. Elevation of intracellular calcium activates a calmodulin/calmodulin kinase system, which induces c-fos expression.

An ion channel that gates ions in response to cytosolic levels of a second messenger triggered by target receptor ligand binding is utilized in the present system. In a preferred aspect of the invention, the present system exploits, and the cells therefore produce, an ion channel that is gated by a cyclic nucleotide such as cyclic AMP (cAMP) and cyclic GMP (cGMP) (for general review see Zagotta, An. Rev. Neurosci. (1996) 19: 235-263). In their natural environment, cyclic nucleotide-gated channels (CNG channels) act as biological signal transducers, converting sensory input, such as light and smell, to electrical signals for processing by the central nervous system. In the olfactory system, for instance, odorant molecules bind to receptors in the olfactory epithelium which are positively linked via a second messenger system, i.e. are linked positively via a G-protein to adenylyl cyclase (AC). Stimulation of AC leads to increases in intracellular levels of the cAMP, which directly binds to and activates CNG channels located in the plasma epithelium. These CNG channels are cation nonselective

channels, fluxing both monovalent cations such as  $\text{Na}^+$ , and divalent cations including  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  the opening of which leads to cellular depolarization and ultimately neurotransmitter release.

- 5 Suitable for use in the present system are any of the CNG channels that have been identified in a variety of tissues( for a review, see Biel et al, Trends Cardiovasc Med, 6(8):274,1996. In one embodiment of the invention, the CNG channel is a retinal CNG channel. The retinal CNG channels typically are much more sensitive to activation by cGMP than cAMP. Specific retinal CNG channels useful in the present system include the cloned human retinal CNG described by  
10 Dhallan et al. (1992), J. Neuro Sci., 112: 3248-3256, and species homologs thereof.

In other, more preferred embodiments of the invention, the CNG channel is an olfactory CNG channel. The olfactory CNG channels are activated with high potency and efficacy by both cAMP and cGMP and are significantly more permeable to  $\text{Ca}^{2+}$  than are retinal CNG channels.

- 15 Suitable for use in the present system are the olfactory CNG channels cloned from a number of species including cow (Lydwig et al. (1990), FEBS, 270: 24-29), rat (Dhallan et al. (1990), Nature, 347: 184-187), catfish (Goulding et al. (1992), Neuron, 8: 45-58) and mouse (Ruiz et al. (1996), J. Mol. Cell. Cardiol., 28: 1453-1461).

- 20 The CNG channels are formed structurally as heterodimers of  $\alpha$ - and  $\beta$ -subunits (Dhallan et al. (1990), Nature, 347: 184-187). The  $\beta$ -subunit, when exogenously expressed in *Xenopus* oocytes or a human embryonic kidney cell line (HEK293), forms functional CNG channels, while the  $\alpha$ -subunit does not (Bradley et al. (1994), PNAS, 91: 8890-8894). However, co-expression of the  $\alpha$ - and  $\beta$ -subunits results in formation of heteromeric CNG channels with ion  
25 permeability, pharmacology and cyclic nucleotide selectivity different from the homomeric channel formed by the  $\beta$ -subunit alone, and more similar to the properties seen in the native channel (Bradley et al. (1994), PNAS, 91: 8890-8894).

- Embodiments of the present invention therefore embrace CNG channels that are homomeric  
30 CNG channels consisting only of functional  $\alpha$  subunits, as well as heterodimeric CNG channels that incorporate both  $\alpha$  and  $\beta$  subunits.



In further embodiments of the invention, the CNG channel is one that retains the characteristic function of gating ion in response cyclic nucleotide binding, but is altered structurally to modify such properties as ion permeability and cyclic nucleotide binding affinity. Mutation studies have identified the molecular basis of ion selectivity, including  $\text{Ca}^{2+}$  permeability, cyclic nucleotide selectivity, and modulation of channel function by  $\text{Ca}^{+}$ /calmodulin and transition metal divalent cations. Thus, much is known about the molecular basis of channel properties which can be exploited in the optimization of these proteins for specific purposes. For example, Warnum et al. (1995) Neuron, 15(3): 619-625, have demonstrated that the cyclic nucleotide selectivity was significantly altered by the substitution of a nonpolar residue for an aspartic acid residue in the cyclic nucleotide binding domain. Still other such modifications to CNG channels are received by Biel et al, supra. Thus, the ability of a cyclic nucleotide to gate the ion channel can be manipulated while retaining functional properties useful in the present system.

In its preferred form, the present assay system exploits cells that produce ion channels that regulate ion flow in response to altered cytosolic levels of a cyclic nucleotide, such as cAMP and cGMP. These CNG channels are capable of reporting activity at any receptor target that is coupled to a second messenger system that modulates cytosolic levels of a cyclic nucleotide. Receptor targets that are naturally coupled to such a second messenger system are the G-protein coupled receptors, and the present system is accordingly well suited for identifying ligands of G-protein coupled receptors.

Thus, the present invention provides, in a preferred aspect, a method for screening chemical compounds to identify ligands for G-protein coupled receptors, comprising the steps of:

(a) obtaining a culture of cells that produce (i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target;

(b) incubating the culture of cells with a chemical compound that is a target G-protein coupled receptor ligand candidate; and

(c) determining the effect of the receptor ligand candidate on ion channel activity.

The term "G-protein coupled receptor" or "GPCR" refers to a diverse class of receptors that mediate signal transduction via an intracellular second messenger system involving binding to G proteins. Briefly, in this second messenger system, signal transduction is initiated via ligand binding to the GPCR, which stimulates binding of the receptor to the G protein.

- 5 Interaction between the receptor and G protein releases GDP, which is specifically bound to the G protein, and permits the binding of GTP, which activates the G protein. Activated G protein dissociates from the receptor and, depending on the type of G protein, activates or deactivates an effector protein such as adenylyl cyclase or guanylyl cyclase. Thus, in the second messenger system associated with GPCRs, the effector protein regulates the intracellular
- 10 levels of specific intracellular messengers (secondary messengers) including cyclic nucleotide such as cAMP and cGMP, as well as inositoltriphosphate (IP<sub>3</sub>) and diacyl glycerol (DAG).

- GPCRs, which are glycoproteins, are known to share certain structural similarities and homologies (see, e.g., Gilman, A.G., *Ann. Rev. Biochem.* 56: 615-649 (1987), Strader, C.D. et al. *FASEB Journal* 3: 1825-1832 (1989), Kobilka, B.K., et al. *Nature* 329:75-79 (1985) and
- 15 Young et al. *Cell* 45: 711-719 (1986)). Among the G protein-coupled receptors that have been identified and cloned are the muscarinic receptors (Hulme et al. (1990), *Annu. Rev. Pharmacol. Toxicol.*, 30: 633-673), the adenosine receptors (Olah et al. (1995), *Annu. Rev. Pharmacol. Toxicol.*, 35: 581-606), the adrenergic receptors (Hieble et al. (1995), *J. Med. Chem.*, 38: 3415-3444), the lysophosphatidic acid receptor (Thompson et al. (1996), *Mol. Pharmacol.*, 45: 718-723), the NPY receptors (Wan et al. (1995), *Life Sci.*, 56: 1055-1064) and the dopamine receptors (Strange et al. (1996), *Adv. Drug Res.*, 28: 313-351). GPCRs share a conserved structural motif. The general and common structural features of the G
- 20 protein-coupled receptors are the existence of seven hydrophobic stretches of about 20-25 amino acids each surrounded by eight hydrophilic regions of variable length. It has been postulated that each of the seven hydrophobic regions forms a membrane-spanning alpha helix and the intervening hydrophilic regions form alternately intracellularly and extracellularly exposed loops. The third cytosolic loop formed between the transmembrane domains is known to be principally responsible for the selective interaction with G proteins.
- 25
- 30 The variety of GPCRs for which ligands can be identified in accordance with the invention is extensive, and includes receptors for the following ligands: adenosine, cannabinoid, melanocortin, adrenergic, dopamine, serotonin, histamine, muscarinic,

bombesin/neuromedin, cholecystokinin, gastrin, tachykinin, opsin, bradykinin, angiotensin, chemokine, angiotensin, endothelin, neuropeptide Y, calcitonin, corticotropin releasing factor, C5a, C3a, fMLP, opsin, eicosanoid, FSH, galanin, leukotriene, opioid, oxytocin, PAF, vasopressin, glucagon, GLP-1, GLP-2, GIP, PACAP, VIP, secretin, vasotocin, melatonin, latrotoxin, metabotropic glutamate and GABA-B receptors, and pheromones. The method of the present invention can further be exploited to identify ligands for GPCR receptor targets that are so-called "orphan" receptors. Particularly, many GPCRs have been cloned which show insufficient homology to previously characterized GPCRs to readily predict their endogenous ligand. These novel putative receptors represent a large pool of potential therapeutic targets for novel drug discovery.

Furthermore, the method of the present invention can be exploited to identify receptors that are targets for known ligands for which the receptor is unknown or has yet to be identified and cloned. Particularly, cells are constructed in which DNA coding for the putative receptor target is incorporated expressibly, in the manner exemplified herein, and the present assay system is thereafter utilized to identify the receptor-encoding clone that modulates ion channel activity when incubated with the known ligand.

For purposes of discovery drugs to treat human medical conditions, it is herein preferred that the GPCR targets are human GPCRs.

In embodiments of the invention, the present system is exploited to identify ligands of positively coupled GPCRs, in which the G protein is of the Gs type. Examples of positively coupled GPCRs are numerous and include the GLP-2 receptor, the 5HT6 receptor, and the the D1 sub-family of dopamine receptors including D1 and D5. In the second messenger system coupled to these receptors, receptor stimulation, such as would be caused by binding of an agonist ligand, results ultimately in an upregulation of cyclic nucleotide. Ligands having agonist activity at these Gs protein coupled receptors will accordingly trigger a readily detectable influx of ion through the CNG channel.

The present system can be exploited also to identify ligands at positively coupled GPCRs that function as antagonists. This can be achieved, in accordance with embodiments of the invention, by introducing the candidate antagonist either together with, or more preferably prior to introduction of a reference agonist for the receptor, and then determining whether the

antagonist candidate depresses the effect of the reference agonist on ion flux. Reference agonists for a given receptor can be identified using the procedure just described above.

5 In other embodiments of the invention, the present system is exploited to identify ligands for negatively coupled GPCRs, in which the G protein is of the Gi type or the Go type. Examples of negatively coupled GPCRs are numerous and include the edg receptors, the NPY receptors, members of the D2 subfamily of dopamine receptors including D2, D3 and D4, and the 5-HT1 subfamily of 5-HT receptors, including the 5-HT1d receptor. In the second messenger system coupled to these receptors, receptor/binding does not stimulate cyclic  
10 nucleotide production, and hence does not cause ion influx through the channel. Ligand binding events at these negatively coupled GPCRs can nevertheless be determined on the basis of ion flux as described below.

In embodiments of the invention, the present system is exploited to identify ligands that are agonists at negatively coupled GPCRs. This is achieved by treating the cell to upregulate  
15 cyclic nucleotide and thereby stimulate influx of ion through the CNG channel. Such stimulation can be achieved by treating the cell to upregulate cyclic nucleotide artificially and thereby stimulate the influx of ion. Such upregulation can be achieved using effector protein activators. If the effector protein is adenylate cyclase a suitable activator is forskolin (Seamon et al. (1981), J. Cyclic Nucleotide Res., 7(4): 201-224). In stimulating ion flux this way,  
20 ligand candidates can then be identified as agonists by their ability to diminish such ion flow relative to activator alone, either when incubated with the activator, or when introduced before the activator.

In other embodiments of the invention, the present system is exploited to identify ligands that  
25 are antagonists at negatively coupled GPCRs. This can be achieved, in accordance with embodiments of the invention, by first incubating cells either first with antagonist and then with agonist, or with a combination of antagonist and agonist, and then treating the cell with an effector protein activator, such as forskolin, to stimulate ion flux into the cell. Such reference agonists at negatively coupled GPCRs can be identified as just described. The  
30 ligand candidate can then be identified as an antagonist by its ability to modulate the known effect of the effector protein activator on ion flow; particularly, an antagonist will counter the agonist-mediated decrease in ion flux stimulated by the effector protein activator.

It will be appreciated that the present system can most conveniently be applied to identify ligands for GPCRs that are positively coupled, for the reason that such ligands can be identified directly by the effect they have on opening (agonists) or inhibiting agonist-mediated activation of (antagonists) the CNG channel. To exploit this convenience, there is further provided in embodiments of the present invention, a system in which GPCRs that are not positively coupled are rendered positively coupled using recombinant DNA technology. The GPCRs of this type are referred to herein as chimeric GPCRs. These chimeric GPCRs have the structural features of non-positively coupled GPCRs, and the ligand binding properties thereof, but feature a third transmembrane loop that has been altered to reproduce a third transmembrane loop of a Gs-type GPCR. This has the effect of coupling the otherwise negatively coupled GPCR to a Gs-type second messenger system, thereby permitting ligand binding at the chimeric GPCR to be determined with the convenience available for Gs protein coupled receptor ligands.

In other embodiments of the invention, the positive coupling assay format can be retained for GPCRs that are not positively coupled, i.e., for those GPCRs coupled to Gi, Gq, or G11, by genetically altering the cell to produce a chimeric G-protein in the manner described in WO98/16557, the disclosure of which is incorporated herein by reference. Briefly, the properties of positive coupling intrinsic to Gs proteins is conferred by all but about the first 5-30 N-terminal amino acids of the G protein. This N terminal region of the G-protein constitutes the GPCR binding domain, which can be replaced in the Gs protein by an N terminal domain capable of binding to the non-positively coupled GPCR target selected for screening. The result is a G protein that will bind a negatively coupled GPCR, but will transduce the signal as would a positively coupled GPCR and thereby allow stimulation at that negatively coupled receptor to be reported more conveniently by the influx of ion at the CNG channel.

In still other embodiments of the invention, the present system can be exploited using cells that produce a G protein known as the promiscuous G protein, or G 16. This G protein is capable of mediating an upregulation of cyclic nucleotide, and hence an opening of the CNG channel, regardless of whether the GPCR target is ordinarily coupled positively to the Gs protein, or negatively to the Gi protein. The cloning and incorporation of the promiscuous G protein into cells is described in WO97/48820, the contents of which are incorporated herein by reference.

Thus, GPCRs can be classified as being positively or negatively coupled to the effector protein. Agonist binding to a positively coupled GPCR will result in an increase in the intracellular level of the secondary messenger produced by effector protein, whereas agonist binding to negatively coupled GPCR will result in a decrease in the level of a secondary messenger. Examples of effector proteins include adenylyl cyclase, guanylyl cyclase and phospholipase C.

To identify target receptor ligands in accordance with the invention, approaches established in related arts can readily be applied. In particular, accumulation of cytosolic cation can be revealed using commercially available dyes that bind the target cation to yield a detectable result, for example either an observable change, for instance in colour, or an altered wavelength detectable by spectrophotometry, such as fluorescence detectable by a fluorimeter. In a particularly preferred embodiment of the invention, the ligand binding event is revealed by detection of the calcium ion, and dyes that fluoresce upon chelating calcium are exploited to report that detection. To prepare cells for screening in this fashion, cells are pre-loaded with the dye in an established manner, so that colour formation results following subsequent binding, in the screen, of a target receptor ligand. Suitable calcium detection dyes include Fura-2, Fluo-3, Fura-Red, Bapta-AM, Quin-2, Calcium Green, and the protein, apoequorin. Detection of such dyes can be achieved using a fluorescence detector, such as a Fluoroskan.

As an alternative to measuring ion flux, CNG channel activity can be determined indirectly by measuring consequences of ion flow, such as by measuring release of acetylcholine from intracellular stores.

When, in accordance with preferred embodiments, the applied screening protocol relies on the formation of a cyclic nucleotide such as cAMP, it is desirable to load cells with a cyclic nucleotide phosphodiesterase inhibitor before incubations are commenced. Such agents prevent the formed cyclic nucleotide from being recycled to precursor products in the cytosol, and can therefore sustain the calcium influx response under detection. Examples of such inhibitors include 3-isobutyl-1-methylxanthine (IBMX) and 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone sold as Rolipram, which prevent reversion of cAMP, and the calcium/calmodulin-dependent phosphodiesterase inhibitor, vinpocetin.

For use in the present system, the present invention further provides cells that are genetically adapted so that activity at a receptor target is coupled to activity at an ion channel, so that altered ion channel activity reports a ligand binding event at the receptor. The cells of the present invention are therefore characterized by the production of the receptor target, the ion channel and a second messenger system that transduces a binding event at the receptor into a detectable activity at the channel.

As used herein the term "genetically adapted" is used with reference to a cell which has been modified by the intervention of man such that the expression of one or more endogenous genes of a host cell has been altered to establish a pattern suited to assessing ion flow, or an intracellular event influenced by ion flow, on ligand binding to a receptor target. This can be achieved, for instance, simply by activating expression of one or more of an endogenous ion channel or a G-protein coupled receptor, by intervention at the genomic level, as disclosed in WO 9412650 which is incorporated herein by reference. Briefly, homologous recombination or targeting can be used to replace or disable the regulatory region normally associated with the selected gene, which results in a pattern of regulation different from that of the parent cell. Alternatively, the level of expression of one or more of a receptor target, an ion channel protein or a second messenger system element, such as a G protein, can be increased by transiently or stably transfecting a cell with heterologous nucleic acid expressing these proteins.

As used herein, "heterologous DNA" includes DNA that does not occur naturally as part of the genome in which it is present, and DNA found in a location or locations in the genome that differs from that in which it occurs in nature. Heterologous DNA is not endogenous to the cell into which it is introduced, but has been obtained from another cell or synthesized de novo, and introduced exogenously. Generally, although not necessarily, such DNA encodes proteins that are not normally produced by the recipient cell in which that DNA is expressed.

In a preferred aspect, the present invention thus further provides a cell, or culture thereof, that has been genetically adapted to produce (i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion channel activity is modulated in response to ligand interaction with the G-protein coupled receptor target.

In embodiments of the invention, the GPCR is a Gs-coupled GPCR or a GPCR that is other than a Gs-coupled GPCR but has been converted to a Gs-coupled GPCR by engineering of its G-protein binding site to a Gs-coupled GPCR; the CNG channel protein is an olfactory CNG protein, and the second messenger system incorporates a Gs protein or a promiscuous G protein. In still more specific embodiments, the cell expresses at least one of such proteins from heterologous DNA coding therefor. Suitably, at least two of such proteins are expressed from heterologous DNA; for instance the GPCR and the CNG channel. Alternatively, the cell can produce all three such proteins from heterologous DNA.

To provide such cells, in accordance with one embodiment of the invention, a host cell that naturally (endogenously) expresses a CNG channel, such as an olfactory or retinal host cell, is pre-screened to establish if it expresses a GPCR target. If it is established that the CNG channel-expressing cell naturally produces the GPCR target, and the endogenous second messenger system is functional, such a cell can be used to screen for receptor ligands using the method of the invention. The CNG channel-producing cell can be assessed for expression of a GPCR target using methods common in the art, for example, using a competition based assay using labelled and non-labelled GPCR reference ligands. If the CNG channel-producing host cell does not produce a GPCR target, heterologous DNA coding for the GPCR target can be introduced therein using standard techniques of molecular biology.

Alternatively, a cell that naturally expresses the GPCR target can be transformed with heterologous DNA encoding a CNG channel, for use in the present system.

In specific embodiments of the invention, a host cell is transformed with heterologous DNA coding for each of the CNG channel and the GPCR target.

Host cells useful in the present system include the various eukaryotic cells such as yeast, *Aspergillus*, insect, *Xenopus*, avian and particularly mammalian cells. Suitable cells include Chinese hamster ovary (CHO) cells for example of K1 lineage (ATCC CCL 61) including the Pro5 variant (ATCC CRL 1281); the fibroblast-like cells derived from SV40-transformed African Green monkey kidney of the CV-1 lineage (ATCC CCL 70), murine L-cells, murine 3T3 cells (ATCC CRL 1658), murine C127 cells, human embryonic kidney cells of the 293 lineage (ATCC CRL 1573), human carcinoma cells including those of the HeLa lineage (ATCC CCL 2), and neuroblastoma cells of the lines IMR-32 (ATCC CCL 127), SK-N-MC (ATCC



HTB 10) and SK-N-SH (ATCC HTB 11). In specific embodiments, the host cell is a HEK 293 cell.

A variety of gene expression systems have been adapted for use with these hosts and are now commercially available, and any one of these systems can be selected to drive expression of the receptor-encoding DNA. Expression vectors may be selected to provide transformed cell lines that express the receptor-encoding DNA either transiently or, more desirably, in a stable manner. For transient expression, host cells are typically transformed with an expression vector harbouring an origin of replication functional in a mammalian cell. For stable expression, such replication origins are unnecessary, but the vectors will typically harbour a gene coding for a product that confers on the transformants a survival advantage, to enable their selection such as a gene coding for neomycin resistance in which case the transformants are plated in medium supplemented with neomycin.

These systems, available typically in the form of plasmid vectors, incorporate expression cassettes the functional components of which include DNA constituting expression controlling sequences, which are host-recognized and enable expression of the receptor-encoding DNA when linked 5' thereof. The systems further incorporate DNA sequences that terminate expression when linked 3' of the receptor-encoding region. Thus, for expression in the selected mammalian cell host, there is generated a recombinant DNA expression construct in which the receptor-encoding DNA is linked with expression controlling DNA sequences recognized by the host, and which include a region 5' of the receptor-encoding DNA to drive expression, and a 3' region to terminate expression.

Included among the various recombinant DNA expression systems that can be used to achieve mammalian cell expression of the receptor-encoding DNA are those that exploit promoters of viruses that infect mammalian cells, such as the promoter from the cytomegalovirus (CMV), the Rous sarcoma virus (RSV), simian virus (SV40), murine mammary tumour virus (MMTV) and others. Also useful to drive expression are promoters such as the LTR of retroviruses, insect cell promoters such as those regulated by temperature, and isolated from *Drosophila*, as well as mammalian gene promoters such as those regulated by heavy metals i.e. the metallothionein gene promoter, and other steroid-inducible promoters.

Through the use of the present system, any of the commercially available chemical libraries, including small molecules and peptides, may be usefully screened. In order to facilitate screening of a large chemical compound bank, the method of the invention may be automated, thereby allowing high-throughput screening of compounds, for example,  
5 according to the method disclosed in U.S. patent 5,589,351, which is incorporated herein by reference. Briefly, the method entails the use of multi-well plates containing cells according to the invention which are loaded with a dye which fluoresces when a metal, e.g.,  $\text{Ca}^{2+}$ , ion is bound. A robotic device that can detect fluorescence, such as a Flouroskan, can be used to rapidly process such plates. Using this approach, chemical banks comprising hundreds of  
10 thousands of compounds can be rapidly screened for receptor agonists.

In accordance with another aspect of the invention, the present system is exploited in a "multiplexed" fashion, which accelerates the rate at which a chemical library can be screened for receptor ligands. This is achieved by exposing the ligand candidate simultaneously to a  
15 mixed culture of channel-producing cells, in which a first cell line produces a first receptor target and a second cell line produces a different, second receptor target. So that ion influx can be detected in a way that discriminates between cell types, reporter systems that are unique to the cell type are used. In an embodiment of the invention, this discrimination is achieved by loading the different cell types with different ion chelating dyes that serve as  
20 "signature" dyes for a particular cell type. For dyes that chelate calcium, for instance, specific embodiments of the invention include the use of dyes having a fluorescent signal that increases with influx of calcium, such as Fluo-3, and dyes that have a fluorescent signal that decreases with influx of calcium, such as Fura Red. Detection of fluorescence specific to cells loaded with a particular dye can then be achieved using filters specific for the  
25 wavelength emitted by the given dye.

As in the method described above for single-type cell assays, the multiplexed assays are capable of detecting ligand binding at GPCRs that are either positively coupled or negatively coupled, and can be used to distinguish between agonists and antagonists at a particular  
30 GPCR species. Specific embodiments which demonstrate this ability are provided in the examples herein.

For use in the multiplexed format of the present system, there is provided, in another aspect of the invention, a mixed culture of cells in which each cell is adapted genetically to produce

(i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target; wherein the mixed culture of cells includes a first cell type that produces a first type of G-protein coupled receptor, and a second cell type that produces a second type of G-protein coupled receptor different from said first type of G-protein coupled receptor.

It can be convenient to format the assay by culturing one of the cell lines in the mixed culture as an adherent culture, and the other as a suspension culture. In the alternative, both cultures can be suspension cultures.

With the multiplexed assay format, it will be appreciated that the binding affinity and functional properties of a chemical compound for two or more receptors can simultaneously be determined from a single incubation. This will be helpful not only to identify ligands for a particular receptor target, but also to profile quickly the other receptor binding properties, and liabilities, of the screened compound. It is also within the scope of the present invention to screen two or more chemical compounds simultaneously, i.e., in a single incubation, with two or more receptor types to yield even more information from the present system. Once identified, the target receptor ligand can then of course be selected for subsequent drug development.

The examples below are provided to illustrate the present system. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

**EXAMPLE 1** – Construction of a CNG channel-producing cell

A) Construction of a full-length rat CNG a clone using PCR.

The rat CNG a gene was cloned into a mammalian expression vector as follows.

Four synthetic oligonucleotides were made as follows:

P1 5'-GGCATTCGGATCCAAGCCACCATGATGACCGAAAAATCCAATGGTG-3';

P2 5'-CCAAGGCTCT AGAGTCACTTATGGTTATTCAGCAGCAGTTGG-3';

P3 5'-GAGTTCTTTGACCGCACTGAGACA-3' and

P4 5'-GCATTCCAGTGGATGATGACCAAG-3'.

The two oligonucleotides P1 and P2 incorporated the start codon and stop codon for the rat CNGa cDNA, (Dhallan et al. (1990), Nature, 374:184-187) respectively. The two oligonucleotides P3 and P4 corresponded to nucleotide sequences within the rat olfactory CNGa open reading frame. The four primers were used to amplify a full-length cDNA encoding the rat CNGa channel using standard RT-PCR procedure. Briefly, reverse transcription (RT) reactions were performed as follows: 25 pmoles of random primers for the 5' end, and 25 pmoles of P2 were incubated, in a solution containing 10 mM KCl, 50 mM Tris-HCl pH 8.3, 3.0 mM MgCl<sub>2</sub>, 0.5 mM of each deoxyribonucleoside triphosphate, 10 mM DTT, 200 units Superscript II Reverse Transcriptase (BRL) and 5 µg rat total hippocampus RNA in a total volume of 20 µl. The conditions for the RT reaction were 1 hour at 42°C and 95°C for 5 minutes. Aliquots (2 µl) of the reverse transcribed RNA samples were used in PCR reactions using the Vent Polymerase (New England Biolabs) according to the manufacturers recommended procedure. Initially two PCR reactions were performed, one reaction using oligonucleotide primers P1 and P4 and another reaction using P2 and P3. These primers sets amplify two overlapping cDNA fragments which together encode a full-length CNGa cDNA. PCR reaction was carried out as follows: 1 minute at 94°C followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, and finally 72°C for 2 minutes. An aliquot of the PCR reaction was electrophoresed on a 1% agarose gel, appropriate bands excised and purified using QIAquick gel extraction kit (Qiagen). To construct a full-length CNGa cDNA the two overlapping PCR products were combined and used as template in a PCR reaction using oligonucleotide primers P1 and P2. The PCR reaction was done as follows: 1 minute at 94°C followed by 30 cycles at 94°C for 30 seconds, 55°C for 1 minute and 72°C for 3 minutes, and finally 72°C for 2 minutes. An aliquot of the PCR reaction was electrophoresed on a 1% agarose gel and the expected 2 kilobase band was excised and purified using QIAquick gel extraction kit (Qiagen). The full-length CNGa cDNA was initially cloned into the vector pCR-Blunt (Invitrogen). Subsequently, the CNG cDNA was excised from pCR-Blunt using restriction enzymes BamHI and XbaI and cloned into the mammalian expression vector pcDNA3.1/Zeo (Invitrogen). The nucleotide sequences of the CNGa clone was determined using an ABI 377 automated sequencer. The sequence agreed with that previously published by Dhallan et al., supra, and Genbank Acc. No. X55519.

## B) Transient transfection in HEK 293 cells.

For transient transfections, cells were plated at a  $5 \times 10^5$  cells per 100 mm plate. The following day at 60-70% confluence, the cells were transfected with 2.5 ug CNG cDNA cloned into pcDNA 3.11 Zeo as above, using the standard lipofectamine transfection protocol (Gibco BRL, 18324-012). On the second day, the cells were plated, in triplicate on 96 well poly-D-Lysine coated plates. On day three, the cells were assayed for calcium uptake using a Fluoroskan.

10 C) Detection of  $\text{Ca}^{2+}$  flux in CNG channel-producing cells

A HEK 293 cell line stably expressing the CNG channel was plated in triplicates on poly-D-Lysine coated plates at approximately  $1 \times 10^5$  per well 2 days prior to day of assay. On the day of the assay, plates were washed 1x with 200  $\mu\text{l}$   $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free buffer (145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES pH 7.2; 300 mOsm) per well. The buffer was removed; cells were pre-loaded with dye by adding 50  $\mu\text{l}$  per well of Fluo-3-AM, and the plates were incubated for 1 hour at RT in the dark. After 1 hour the dye was removed, 200  $\mu\text{l}$  of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free buffer was added per well and the plates were incubated for a further 30 min at RT in the dark. Subsequently, the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free buffer was removed and replaced with 45  $\mu\text{l}$  of  $\text{Mg}^{2+}$ -free buffer containing calcium ion (142 mM NaCl, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , 10 mM glucose, 10 mM HEPES pH 7.2; osmolality = 300 mOsm). A reading was taken using a Fluoroskan (T=0 time). Compounds studied were made up in a concentrated stock using the appropriate solvent, (for example, DMSO for forskolin,  $\text{dH}_2\text{O}$  for 8-Br-cGMP, 8-Br-cAMP and diluted to 4X final concentration and dissolved in 15  $\mu\text{l}$   $\text{Mg}^{2+}$ -free buffer), added to the wells and further readings were taken at T=1 minute, T=5 minute etc. using the Fluoroskan.

Results of the study with transient cells are shown in Figure 1 and illustrate clearly that calcium ion flux is detected in CNG -transformed cells (filled shapes) when treated with either a cyclic nucleotide (8Br-cGMP) or an adenylate cyclase activator (forskolin), but not in mock transfected, similarly treated cells. Figure 2 illustrates results with cells stably producing the CNG channel, at increasing concentrations of 8-Br-cGMP (panel A), 8-Br-cAMP (panel B) and forskolin (panel C).

**EXAMPLE 2** - Incorporation of GPCR into a CNG channel-producing cell**A) Integration of the human 5HT6 gene, as GPCR**

HEK 293 stable cell line expressing the rat olfactory CNG channel was produced from transiently transfected cells in the manner described in example 1, with the following additional steps. On day 3, cells were plated 1:10 and 1:20 in 150 mm plates in DMEM + 10 % FBS + 400 U/ml Zeocin (Invitrogen). Zeocin selection was maintained for approximately 17 days at which point individual colonies are picked and expanded. When sufficient quantities of cells are grown for each colony, cells were plated on 96 well poly-D-Lysine coated plates in triplicates. Approximately 48 hours later the cells were assayed for calcium uptake using a Flouroskan. Thereafter, in preparation for transfection with a cDNA encoding the 5HT6 receptor, the cells were plated out a density of approximately  $1.8 \times 10^6$  cell on a 100 mm plate in MEM with 10% FBS, 1% glutamine and 400 ug/ml Zeocin (Invitrogen) and left to attach for 24-48 hours in a 37°C CO<sub>2</sub> incubator.

Subsequently, 2 µg of a lipofectamine 5HT6/cDNA complex was prepared as follows. The 5HT6 receptor gene was cloned using standard PCR, from fetal brain cDNA library. The sequence agreed with the published nucleotide sequence (Genbank L41147 and J. Neurochem. (1996), 66(1): 47-56). The gene was inserted into the EcoR1/Xba1 multiple cloning sites of pcDNA3 (Invitrogen). 2µg of pcDNA/5HT6 were diluted in 240µl serum-free OPTI-MEM. 22µl lipofectamine was prepared in 240µl OPTI-MEM. The 2 solutions mixed gently and incubate at room temperature for 30-45 minutes. Prior to addition of the DNA/lipofectamine complex to a 100 mm plate, cells were washed once with serum-free MEM and 5 ml serum/antibiotic-free MEM was added.

When cell attachment had taken place 480µl of serum-free OPTI-MEM containing the 2µg of 5-HT 6 cDNA and 22µl of lipofectamine was added, dropwise to the plate. The plate was then gently swirled and left to incubate for 5 hours at 37°C in the CO<sub>2</sub> incubator. After 5 hours, the media was removed and fresh MEM with 10% FBS, 1% glutamine and 400µg/ml Zeocin was added. The cells were allowed to recover overnight at 37°C.

Cells were then trypsinized after washing with 1XPBS and split into 150 mm plates at various dilutions (to ensure well isolated colonies). Cells were allowed to attach to the plates for 24 hours. To select for cells containing both the olfactory CNG channel and the 5HT-6

receptor, the following day the media was changed to MEM containing 10% FBS, 1% glutamine, 800-1000µg/ml G418 (GIBCO/BRL) and 400µg/ml Zeocin. Media was thereafter changed every 48-96 hours, depending on cell death. After a sufficient selection time (approximately 2 weeks), distinct colonies containing both the CNG channel and the 5HT6 receptor appeared on the plate.

Stable cell lines expressing CNG channel and 5HT6 receptor cDNA were cloned using standard procedures. Briefly, sterile cloning rings were placed over colonies and 50µl 1X Trypsin was added to the side of each ring to trypsinize cells for approximately 1-2 minutes. 50µl growth antibiotic-free media was added and pipetted several times. Cells were transferred to 24-well dishes containing 0.5 ml media. The following day the media was removed and MEM media containing 10% FBS, 1% glutamine, 400µg/ml Zeocin and 800-1000µg/ml G418 added.

#### B) *Integration of the human dopamine D1 receptor, as GPCR*

Integration the human D1 receptor into the HEK 293 stable cell line expressing the rat olfactory CNG channel was achieved in the manner described above for the 5HT6 producing cells. The D1 receptor gene was cloned using standard PCR and hybridization screening procedures applied to a human genomic library. The sequence agreed with the published nucleotide sequence (Genbank accession number X55758 and Nature, 1990,347(6288):80-83). The gene was inserted into the EcoRI/XbaI multiple cloning sites of pcDNA3. Three µg of pcDNA3/D1 were diluted in 240 µl of serum-free OPTI-MEM. Transfection and selection were then performed as described above for the 5HT6/CNG stable cell line.

#### EXAMPLE 3 Screening for 5HT6 receptor ligands

To prepare for screening of 5HT6 receptor ligand candidates, the HEK293 cell line stably expressing both the r CNG channel and the h5HT6 receptor was plated in triplicate on poly-D-Lysine coated plates at approximately  $3 \times 10^5$  cells/mL two days prior to assay. On the day of the assay, plates were washed once with 200 µl of CNG buffer (142 mM NaCl, 5mM KCl, 2 mM  $\text{CaCl}_2$ , 10 mM glucose, 10mM HEPES pH7.2; osmolality = 300mOsm) per well. The

buffer was removed, and 35  $\mu$ M of Fluo-3-AM dye (Molecular Probes) was added per well in a volume of 50  $\mu$ L. The plates were incubated for 1 hour at room temperature in the dark. After 1 hour, the dye was removed and cells were washed twice with 200  $\mu$ L of CNG buffer. Subsequently, 200  $\mu$ L of CNG buffer was added to each well and the plates were incubated for a further 10 minutes at room temperature in the dark.

*A) Agonist screening*

Calcium influx was measured following 10 minutes of incubation with 15  $\mu$ L of 10  $\mu$ M each of the 5HT<sub>6</sub> receptor ligands 5-hydroxytryptamine (5-HT), 5-carboxytryptamine (5-CT) and 5-methoxytryptamine (5-MeOT). Fluorescence was detected by Fluoroskan as noted above, and the results are shown in Panel A of Figure 3. As anticipated, incubation with these agonists of the Gs-type 5HT<sub>6</sub> receptor resulted in stimulation of calcium influx, as revealed by the increased fluorescence relative to controls. Similar results were obtained when the agonists were assessed against CNG-producing cells that transiently produce the 5HT<sub>6</sub> receptor.

*B) Antagonist effect*

Panel B of Figure 3 illustrates the response seen with the noted known antagonists of the 5HT<sub>6</sub> receptor, using the same protocol as with the agonists. It will be noted that, as expected, no stimulation of calcium influx resulted.

**EXAMPLE 4** - Screening for D1 receptor ligands

*A) Agonist screening*

In the manner described in Example 3A for 5HT<sub>6</sub> agonist screening, but using the CNG-producing D1 receptor cell line as obtained in Example 2B, calcium influx was measured following 5 minutes of incubation with each of the D1 receptor agonists (10  $\mu$ M final concentration, 60  $\mu$ L final volume) noted in Figure 4, panel A. As the results show, interaction between the Gs-type D1 receptor the agonists clearly resulted in a significant influx of calcium ion, relative to mock treated controls.



**B) Antagonist effect**

Using the CNG-producing D1 receptor cell line as obtained in Example 2B, the D1 receptor antagonists noted in Figure 4 panel B were assessed for their effect on calcium influx.

Antagonists were added in 15uL volumes, to a final concentration of 10uM. As shown, the antagonist properties of these compounds are revealed by the absence of detected calcium influx.

**C) Antagonist screening**

To identify ligand candidates as functional antagonists, the following protocol was developed, which studies the ability of an antagonist candidate to inhibit calcium influx mediated by a reference agonist.

Cells were dye-loaded and plated in the manner described in Example 3. Cell-borne D1 receptors were then first saturated with the selected antagonist incubating the cells with 50 L of the selected antagonist at 10 M for five minutes. Incubation buffer was then replaced with CNG buffer containing IBMX (0.111mg/mL) in a volume of 30uL, in order to maintain the cytosolic levels of cAMP formed upon subsequent addition of the reference agonist.

An initial t=0 reading was taken on the fluorescence reader (Fluoroskan). Following this read, 15 L of the selected antagonist (10 M final) were added to the pre-treated wells, together with 15uL of dopamine, the reference agonist, at a final concentration of 1 M. Subsequent reads were taken at T=5 minutes, and T=10 minutes.

As shown in Figure 5, results reveal that in the presence of antagonists flupentixol and SCH23390, dopamine response was inhibited (panel B). In panel A cells were not pretreated with antagonist.

**EXAMPLE 5 - Multiplexed Screening**

The examples which follow utilize CNG channel-producing cells that incorporate either no GPCR (for control) or the Gs-coupled 5HT6 or D1 receptors. These cell lines were prepared as described in the preceding examples unless otherwise noted below.

**A) Cell preparation****Dye Loading of HEK293 cells stably expressing rCNG channel**

A HEK293 cell line stably expressing the r CNG channel was plated in triplicate on poly-D-Lysine coated plates at approximately  $3 \times 10^5$  cells/mL 2 days prior to day of assay. On the day of the assay, plates were washed once with 200  $\mu$ L of CNG buffer (142 mM NaCl, 5mM KCl, 2 mM  $\text{CaCl}_2$ , 10 mM glucose, 10mM HEPES pH7.2; osmolality = 300mOsm) per well. The buffer was removed, and 50  $\mu$ M of Fura Red-AM dye (Molecular Probes) in a volume of 50  $\mu$ L was added per well, and the plates were incubated for 1 hour at room temperature in the dark. After 1 hour, the dye was removed and cells were washed with 200  $\mu$ L of CNG buffer, twice. Subsequently, 200  $\mu$ L of CNG buffer was added to each well and the plates were incubated for a further 15 min at room temperature in the dark. Subsequently, the CNG buffer was removed and replaced with 45  $\mu$ L of HEK293 cells stably expressing both the rCNG channel and the hD1 receptor (See below).

**15 Dye Loading of HEK293 cells stably expressing both rCNG channel and h5HT6 receptor:**

A HEK293 cell line stably expressing both the r CNG channel and the h5HT6 receptor was plated in triplicate on poly-D-Lysine coated plates at approximately  $3 \times 10^5$  cells/mL two days prior to day of assay. On the day of the assay, plates were washed once with 200  $\mu$ L of CNG buffer (142 mM NaCl, 5mM KCl, 2 mM  $\text{CaCl}_2$ , 10 mM glucose, 10mM HEPES pH7.2; osmolality = 300mOsm) per well. The buffer was removed, 50  $\mu$ M of Fura Red-AM (Molecular Probes) dye in a volume of 50  $\mu$ L was added per well, and the plates were incubated for 1 hour at room temperature in the dark. After 1 hour, the dye was removed and cells were washed twice with 200  $\mu$ L of CNG buffer. Subsequently, 200  $\mu$ L of CNG buffer was added to each well and the plates were incubated for a further 15 minutes at room temperature in the dark. Subsequently, the CNG buffer was removed and replaced with 45  $\mu$ L of HEK293 cells stably expressing both the rCNG channel and the hD1 receptor (See below).

**Dye Loading of HEK293 cells stably expressing both rCNG channel and D1 receptor**

30 A HEK293 cell line stably expressing both the r CNG channel and the D1 receptor were grown to 90% confluency in a T75 flask. On the day of the assay, cells at this confluency, were washed once with PBS and dislodged by trypsinization. Cells were resuspended in CNG buffer by gentle trituration. Subsequently, 10  $\mu$ M of Fluo-3-AM dye (Molecular

Probes) was added to the cell suspension, and incubated for 1 hour at room temperature in the dark. After 1 hour, cells were centrifuged at 1000 rpm for 5 minutes, and the dye removed. Cells were resuspended in CNG buffer and washed twice by centrifugation at 1000 rpm at room temperature. Following the last wash, cells were resuspended to a density of approximately  $5 \times 10^5$  cells/mL in CNG buffer supplemented with 0.111 mg/mL IBMX (Sigma), and cell clumping was minimized by gentle trituration.

#### B) Agonist Screening

##### 1) Multiplexing cells producing CNG alone with cells producing CNG and D1 receptor

HEK293 cells loaded with Fluo-3-AM which express rCNG and the D1 receptor (see above) were added (45 l) to 96-well plates containing adherent Fura Red-AM loaded HEK293 cells stably expressing the rCNG channel (see above). A reading was taken using the fluorescence reader (Fluoroskan) as T=0. Compounds studied (see Figure 6) were dissolved in the appropriate solvent (for example, dH<sub>2</sub>O for 8Br-cGMP and 8Br-cAMP, and CNG buffer for Dopamine), diluted to four times final concentration in CNG buffer and added in a volume of 15 l to the appropriate wells. Further readings were taken at T=1 minute, T=5 minutes, and T=10 minutes, using the Fluoroskan. Fluoroskan readings were taken using Excitation Filter 485 and Emission Filter 538 for measurement of fluorescence response in cells loaded with Fluo-3-AM, and Excitation Filter 485 and Emission Filter 660 for measurement of fluorescence response in cells loaded with Fura Red-AM.

As shown in Figure 6, cells loaded with Fura Red respond to direct CNG channel activation with a reduction in the mixed culture that are fluorescence signal (panel A), whereas cells in the mixed culture that are loaded with Fluo-3 respond to direct channel activation with an increase in fluorescence signal (panel B). This result is also reflected in results with D1 receptor ligands. As shown in panel C, incubation of D1 agonists with cells producing only the CNG channel, and loaded only with Fura Red, showed no influx of calcium as expected. Also as expected, the D1 receptor antagonists, flupentixol and haloperidol, also failed to stimulate calcium influx, as revealed by the absence of any decrease in Fura Red fluorescence. On the other hand, as shown in Figure 6 panel D, Fluo-3-loaded cells producing the D1 receptor and the CNG channel elicited a significant calcium influx when

incubated with the D1 receptor agonists. As expected, no calcium influx, and accordingly no increase in Fluo-3 fluorescence, was detected when the antagonists flupentixol and haloperidol were incubated.

5    2)    **Multiplexing cells stably producing both rCNG channel and 5HT6 receptor with cells stably producing both rCNG channel and D1 receptor**

HEK293 cells stably expressing both rCNG and the 5HT6 receptor were grown as adherent cultures in 96 well Poly-D-Lysine coated plates, and loaded with the specific calcium indicator Fura Red-AM (50 M). HEK293 cells stably expressing both rCNG and the D1 receptor were grown to 90% confluency in T75 flasks and dislodged by trypsinization. Cells were resuspended in CNG buffer to a density of approximately  $5 \times 10^5$  cells/mL, and loaded in suspension with the calcium specific indicator Fluo-3-AM (10 M). Suspension cells were added together with the adherent cells, and calcium influx was measured following 10 minutes of incubation with a CNG channel activator, 5mM 8Br-cGMP, D1 selective agonists: 10 M Dopamine, 10 M ADTN, 10uM SKF38393, partial agonists, 10 M Apomorphine, 10 M Pergolide, or antagonists: 10uM Flupentixol, 10 M Haloperidol, and 5HT6 selective agonists: 10uM 5HT, 10uM 5CT, 10 M (+)Lisuride, or antagonists: 10 M Clozapine, and 10 M Methiothepin. Calcium influx via 5HT6 receptor activation/inactivation was measured using Excitation Filter 485 and Emmision Filter 660, which are specific for the calcium indicator Fura Red-AM (A, C). D1 receptor activation/inactivation was measured using Excitation Filter 485 and Emmision Filter 538 which are specific for Fluo-3-AM (B,D).

25    As shown in Figure 7, 5HT6 specific agonist activity was observed only in the cell line producing CNG channel and 5HT6 receptor loaded with Fura Red (panel B); these cells did not respond to D1 specific agonists (panel A). The cell line producing CNG channel and D1 receptor loaded with Fluo-3 was responsive only to D1 specific agonist activity (panel B), and was unresponsive to 5HT6 specific agonists (panel D).  
30    Non-selective agonists lisuride and apomorphine activated D1 and 5HT6 receptors in both cell lines (see panels A, B, C, D).

### C) Antagonist Screening

- The compound of interest (10  $\mu$ M final concentration) was added to a combined mixture of two HEK293 cell lines stably expressing rCNG channel and either h5HT6 receptor or hD1 receptor (prepared and dye loaded as described above) in a volume of 10  $\mu$ L. Cells were pretreated with this compound for 5 minutes. Immediately following this pretreatment an initial t=0 reading was taken on the fluorescence reader (Fluoroskan). Following this reading, 10  $\mu$ L of known agonist at a final concentration of 1mM (serotonin for 5HT6 and dopamine for D1) was added to each well. Subsequent reads were taken at T=5 minutes, and T=10 minutes. Excitation filter 485 and Emission filter 538 were used for measurement of fluorescence response in cells loaded with Fluo-3-AM. Excitation filter 485 and Emission filter 660 were used for measurement of fluorescence response in cells loaded with Fura Red-AM. Antagonistic properties of these compounds were assessed based on reversal of either serotonin and/or reversal of dopamine stimulated agonist activity.
- Particularly, HEK293 cells stably expressing both rCNG and the 5HT6 receptor were grown as adherent cultures in 96 well Poly-D-Lysine coated plates, and loaded with the specific calcium indicator Fura Red-AM (50  $\mu$ M). HEK293 cells stably expressing both rCNG and the D1 receptor were grown to 90% confluency in T75 flasks and dislodged by trypsinization. Cells were resuspended in CNG buffer to a density of approximately  $5 \times 10^5$  cells/mL, and loaded in suspension with the calcium specific indicator Fluo-3-AM (10  $\mu$ M). Suspension cells were added together with the adherent cells. Multiplexed cells were pretreated for 5 minutes with 10mM of clozapine (5HT6 selective antagonist), flupentixol (D1 selective antagonist) or propranolol (non-selective antagonist). Following this pretreatment, 1mM of the 5HT6 selective agonist, serotonin (A) or the D1 selective agonist, dopamine (B), was added to each well. Calcium influx via 5HT6 receptor activation/inactivation s measured using Excitation filter 485 and Emmision filter 660, which are specific for the calcium indicator Fura Red-AM (A). D1 receptor activation/inactivation was measured using Excitation filter 485 and Emmision filter 538 which are specific for Fluo-3-AM (B).
- Results are presented in Figure 8, as a percentage reversal of agonist (serotonin (A) or dopamine (B)) response due to the effect of various antagonists. As summarized in panel A, the 5HT-stimulated response could be reversed by clozapine, and partially reversed by

flupentixol. Propranolol had no effect. In panel B, the dopamine-stimulated response is fully reversed by flupentixol and only slightly reversed by propranolol. Clozapine had no effect.

**WE CLAIM:**

1. A method for identifying receptor ligands, comprising the steps of:
  - (a) obtaining a cell that expresses both an ion channel and a target receptor and that possesses an intracellular message system permitting ligand binding at the receptor to trigger measurable modulation of ion flow by the ion channel; and
  - (b) incubating a ligand candidate under receptor-binding conditions with said cell; and
  - (c) determining whether ion flow has been modulated, thereby to identify ligands that bind the target receptor.
2. A method for screening chemical compounds to identify G-protein coupled receptor ligands, comprising the steps of:
  - (a) obtaining a culture of cells adapted genetically to produce (i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target;
  - (b) incubating the culture of cells with a G-protein coupled receptor ligand candidate; and
  - (c) determining the effect of the receptor ligand candidate on the flow of ion through the channel.
3. The method according to claim 2, wherein the cyclic nucleotide gated ion channel is an olfactory cyclic nucleotide gated channel.
4. The method according to claim 3, wherein the G-protein coupled receptor is a Gs-type G-protein coupled receptor.

5. The method according to claim 4, wherein the cyclic nucleotide gated ion channel is an olfactory cyclic nucleotide gated channel.
6. The method according to claim 5, wherein the olfactory cyclic nucleotide gated channel is an alpha homomeric rat olfactory cyclic nucleotide gated channel.
7. The method according to any preceding claim wherein the effect of the receptor ligand candidate on the flow of ion through the channel is determined by measuring the flow of calcium ion through the channel.
8. The method according to claim 7, wherein the flow of calcium ion through the channel is measured using a fluorescent calcium chelating dye.
9. The method according to claim 8, wherein the calcium chelating dye is Fluo-3.
10. The method according to any preceding claim, wherein the culture of cells is incubated simultaneously with at least two different G-protein coupled receptor ligand candidates.
11. The method according to any preceding claim, wherein at least one of the G-protein coupled receptor ligand candidates is a reference ligand for said G-protein coupled receptor.
12. The method according to claim 11, wherein said reference ligand for said G-protein coupled receptor is a reference agonist.
13. A method for screening chemical compounds to identify G-protein coupled receptor ligands, comprising the steps of:
  - (a) obtaining a mixed culture of cells in which each cell is adapted genetically to produce (i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target; wherein said mixed culture of cells includes a first cell type that produces a first type of G-protein coupled receptor and a second cell



type that produces a second type of G-protein coupled receptor different from said first type of G-protein coupled receptor;

- (b) incubating the mixed culture of cells with at least one G-protein coupled receptor ligand candidate; and
- (c) determining the effect of the receptor ligand candidate on the flow of ion through the channels of said mixed culture.

14. A method according to claim 13, wherein, prior to incubation with the G-protein coupled receptor ligand candidate, the first cell culture and the second cell culture are loaded with agents that discriminate between the influx of ion into the first cell culture and the influx of ion into the second cell culture.

15. The method according to any preceding claim, wherein the effect of the receptor ligand candidate on the flow of ion through the channels of said culture is determined by measuring calcium ion influx.

16. The method according to claim 15, wherein said agents are calcium chelating dyes that fluoresce at different wavelengths.

17. The method according to claim 16, wherein the calcium chelating dyes are Fura Red and Fluo-3.

18. A cell that produces (i) a G-protein coupled receptor (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target, wherein at least one of said receptor, said channel protein and said second messenger system is heterologous to said cell.

19. A cell according to claim 18, wherein said G-protein coupled receptor is a human G-protein coupled receptor.

20. A cell according to claim 18, wherein said G-protein coupled receptor is expressed in said cell from heterologous DNA.
21. A cell according to claim 19, wherein said G-protein coupled receptor is a human G-protein coupled receptor.
22. A cell according to any one of claims 18-21, wherein said cell is a mammalian cell.
23. A cell according to any one of claims 18-22, wherein said cyclic nucleotide gated ion channel is an olfactory cyclic nucleotide gated channel.
24. A cell according to claim 23, wherein said olfactory cyclic nucleotide gated channel is the alpha homomeric rat olfactory cyclic nucleotide gated channel.
25. A cell according to any preceding claim, wherein both the cyclic nucleotide gated channel and the G-protein coupled receptor are encoded by heterologous DNA.
26. A mixed culture of cells in which each cell is adapted genetically to produce (i) a G-protein coupled receptor target, (ii) a cyclic nucleotide gated ion channel protein, and (iii) a second messenger system through which ion flow through cyclic nucleotide gated channel is modulated in response to ligand interaction with the G-protein coupled receptor target; wherein said mixed culture of cells includes a first cell type according to any one of claims 19-25, and a second cell type that produces a species of G-protein coupled receptor different from the G-protein coupled receptor species produced by the first cell type.
27. A mixed culture of cells according to claim 26, wherein the G-protein coupled receptor species produced by the first cell type and by the second cell type are both positively coupled G-protein coupled receptors.
28. A mixed culture of cells according to claim 26, wherein the first cell type and the second cell type are loaded with ion chelating dyes that are detectable at different wavelengths.

29. A mixed culture of cells according to claim 28, wherein the first cell type is loaded with Fluo-3.
30. A mixed culture of cells according to claim 29, wherein the second cell type is loaded with Fura Red.

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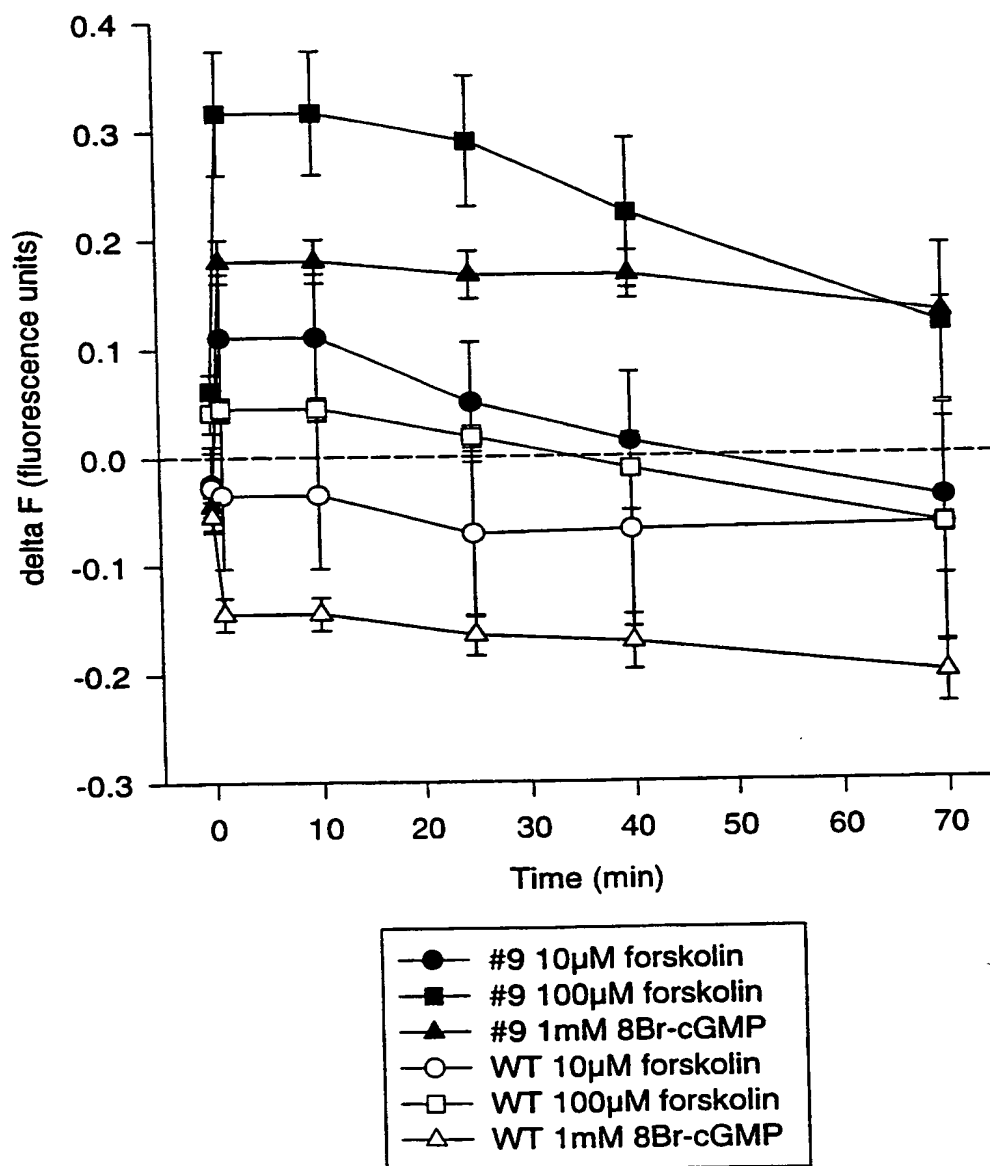


Figure 1

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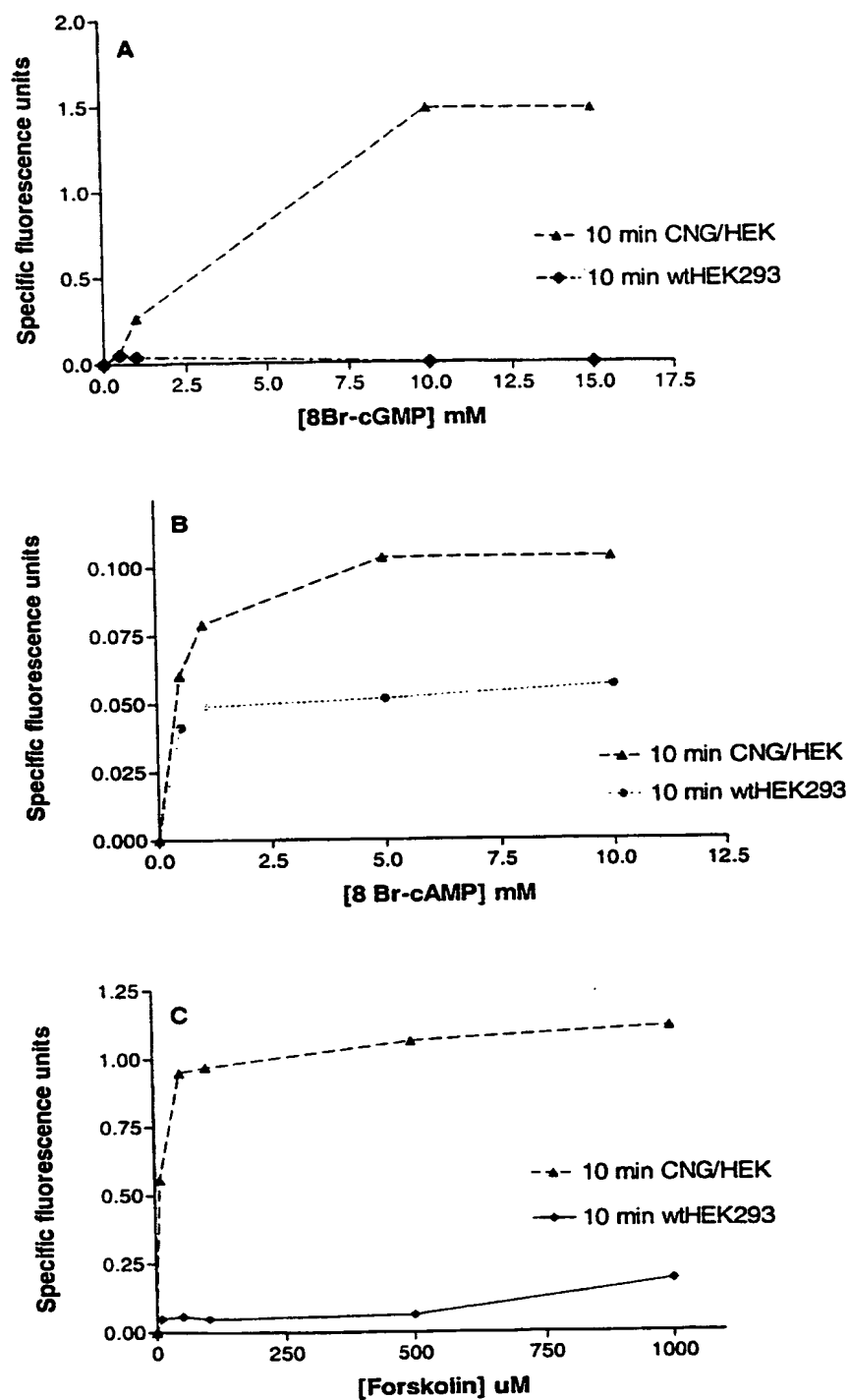


Figure 2

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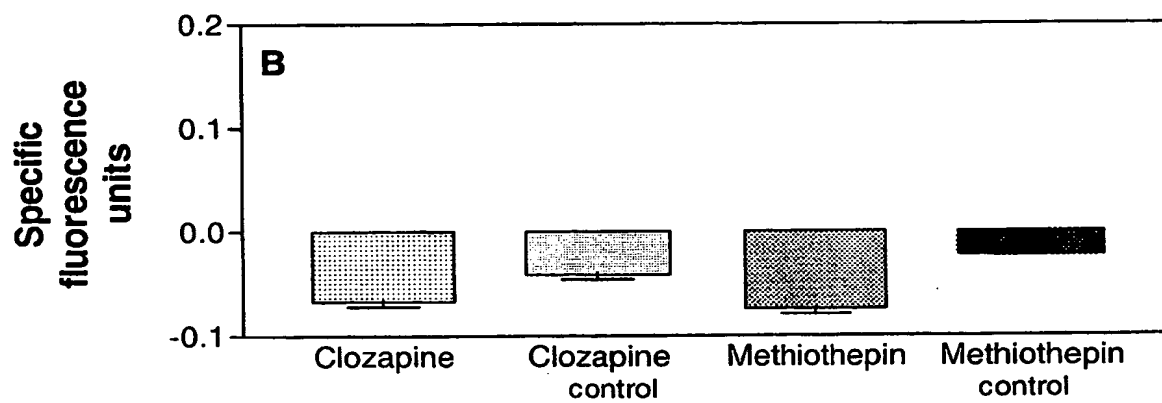
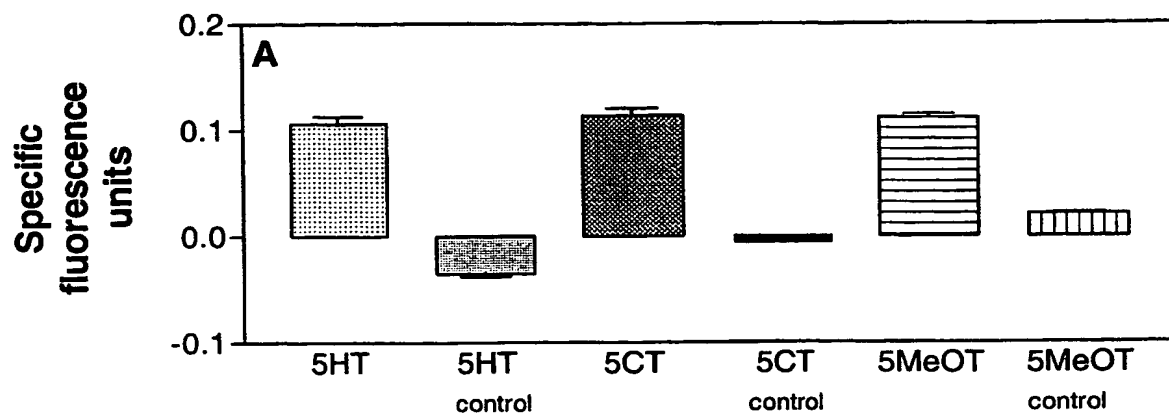


FIGURE 3

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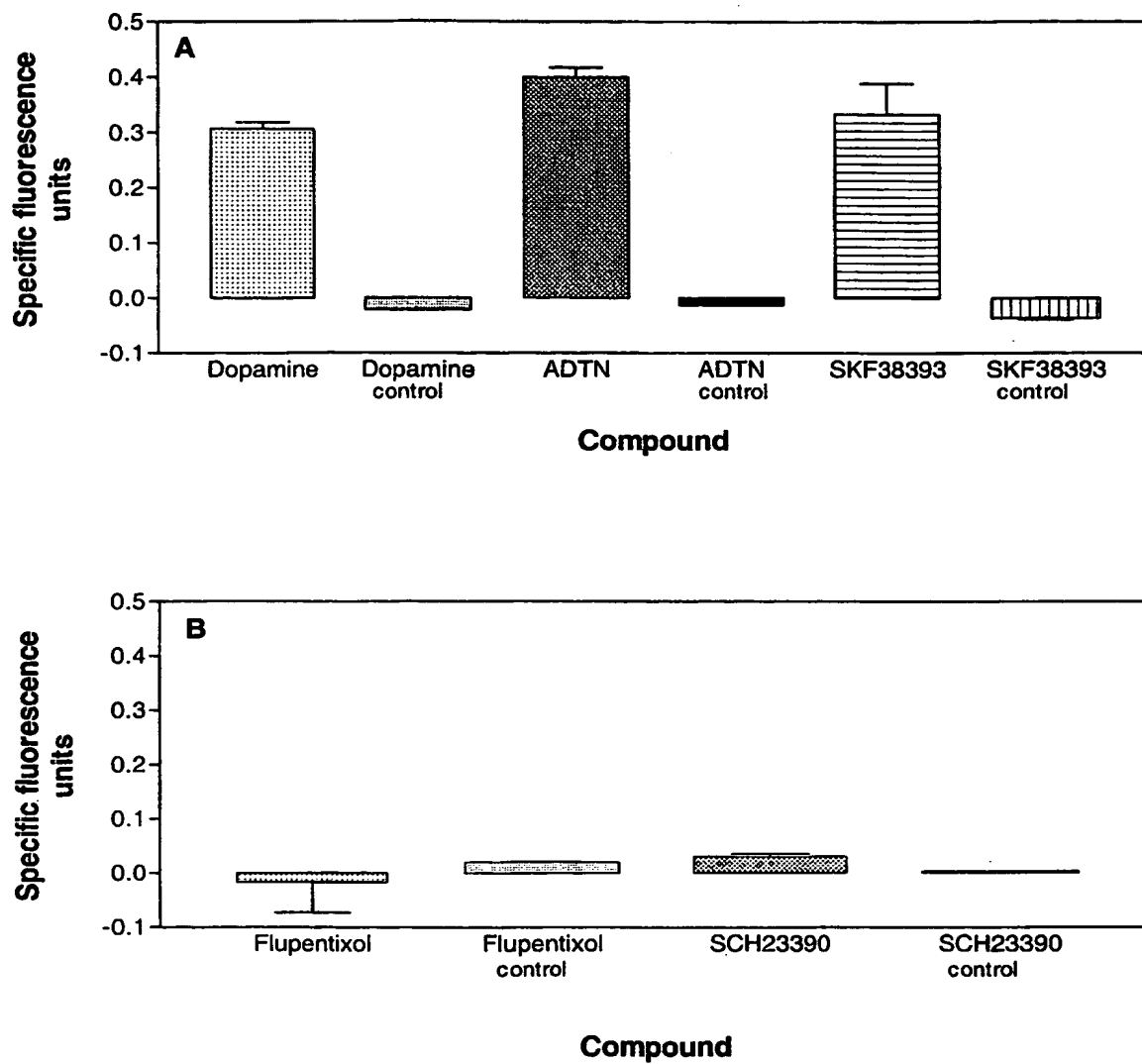


FIGURE 4

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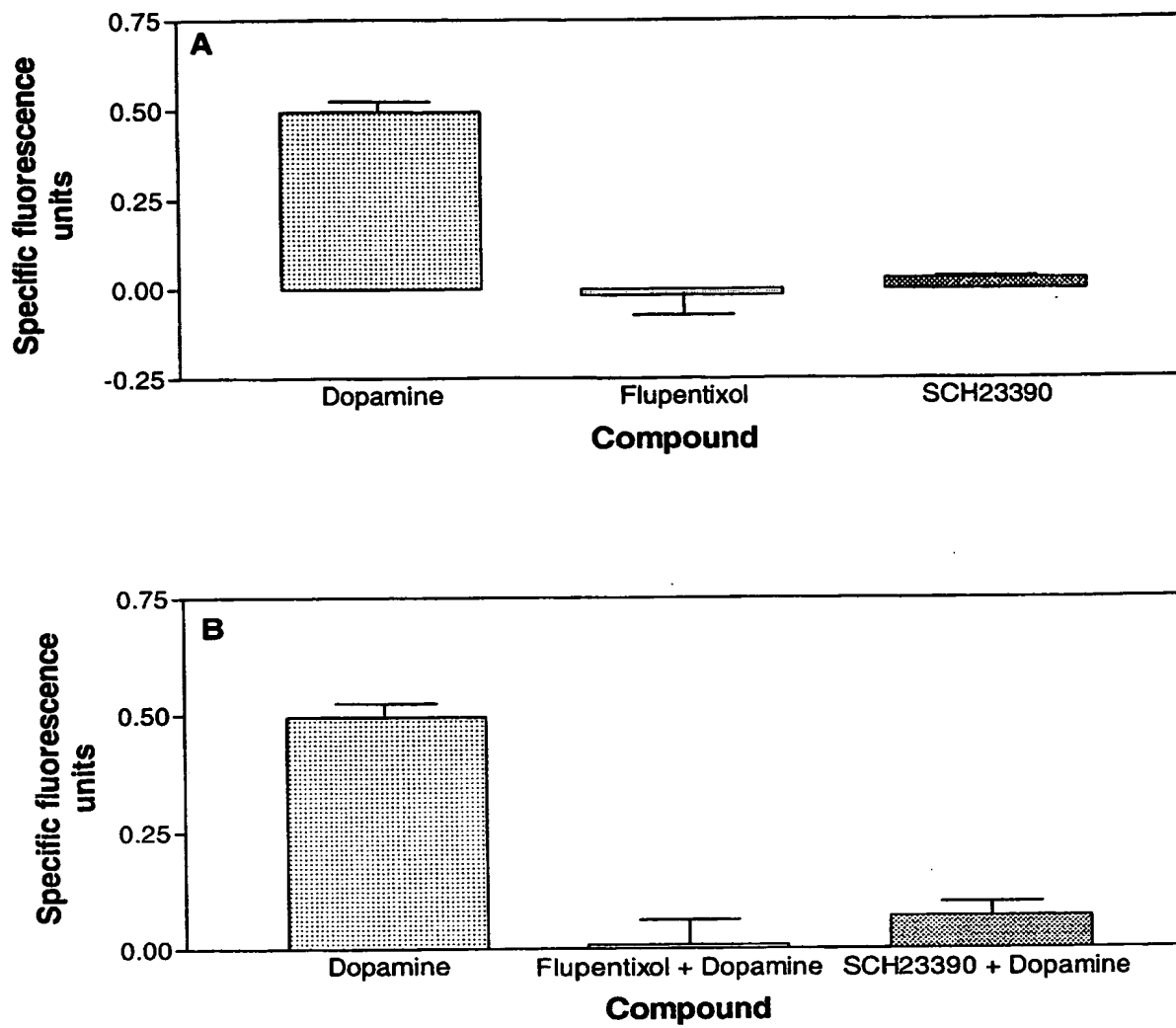


FIGURE 5



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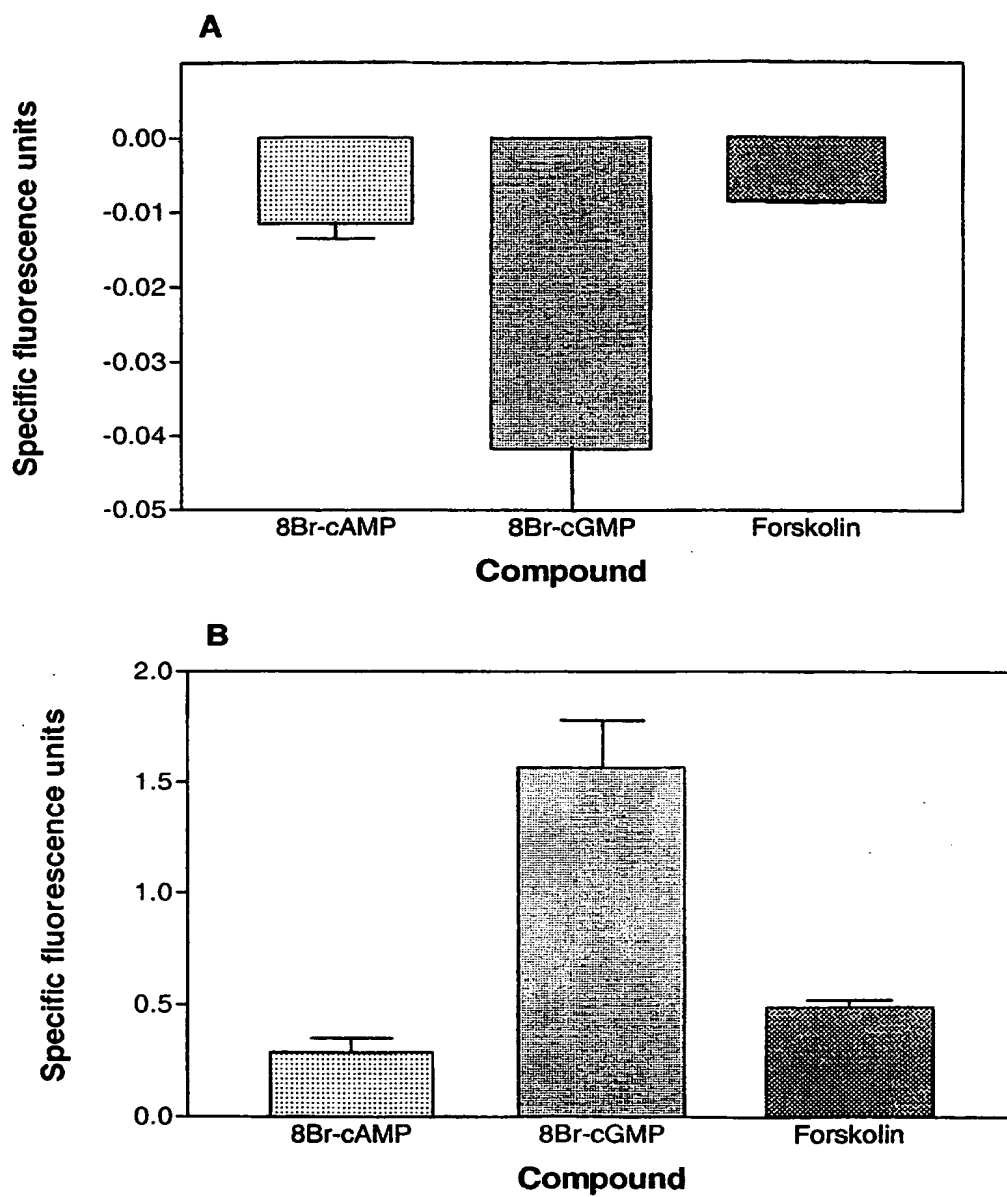


FIGURE 6

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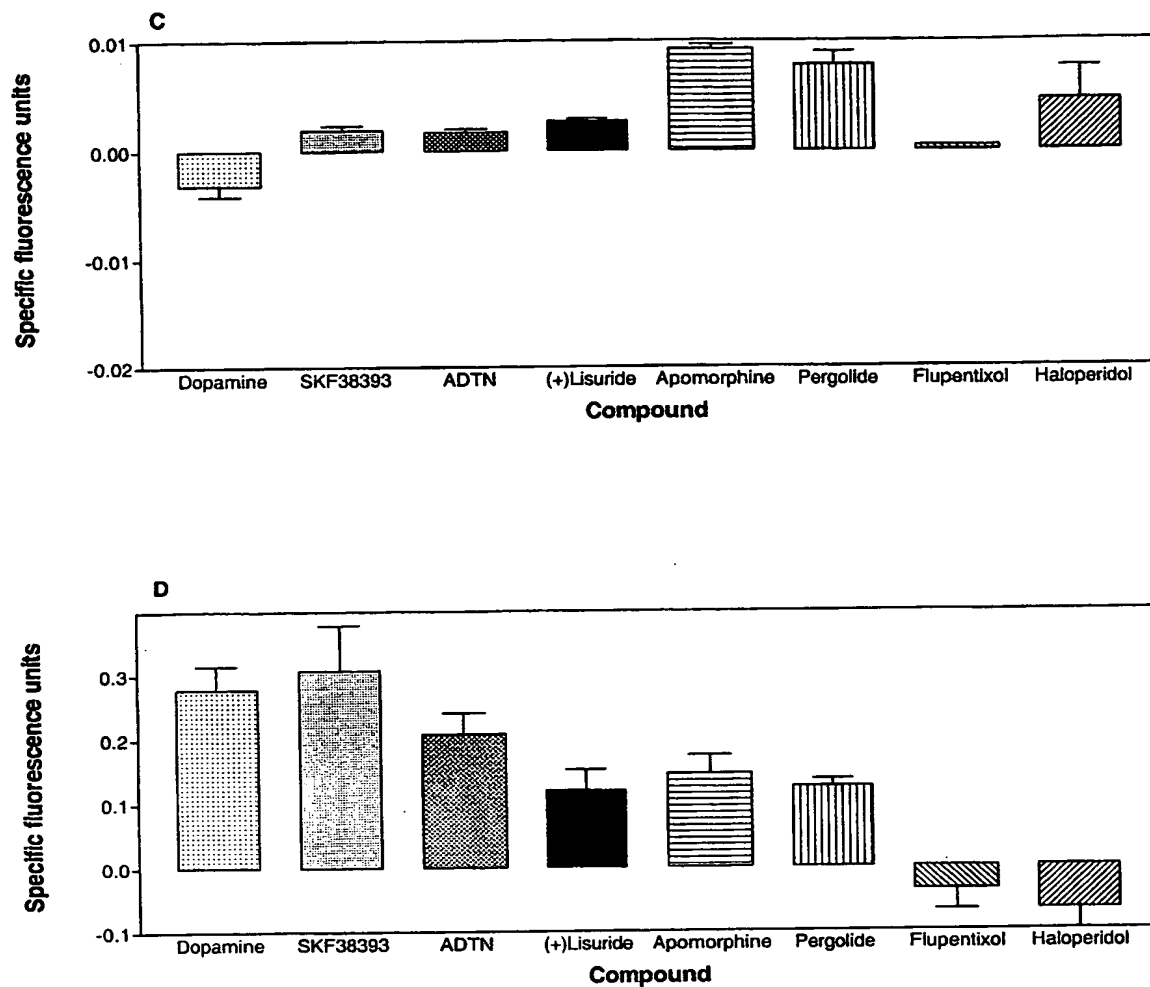


FIGURE 6

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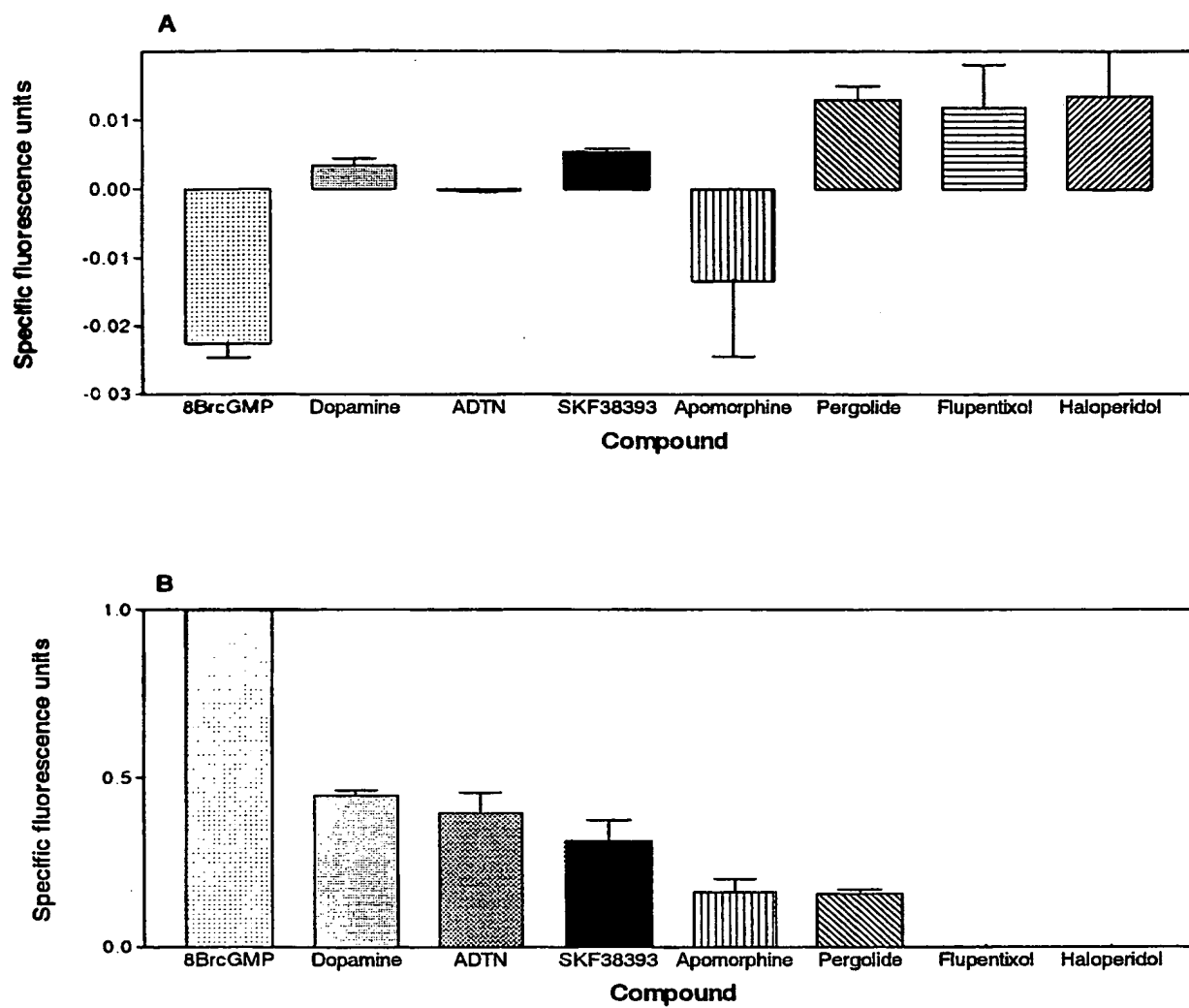


FIGURE 7

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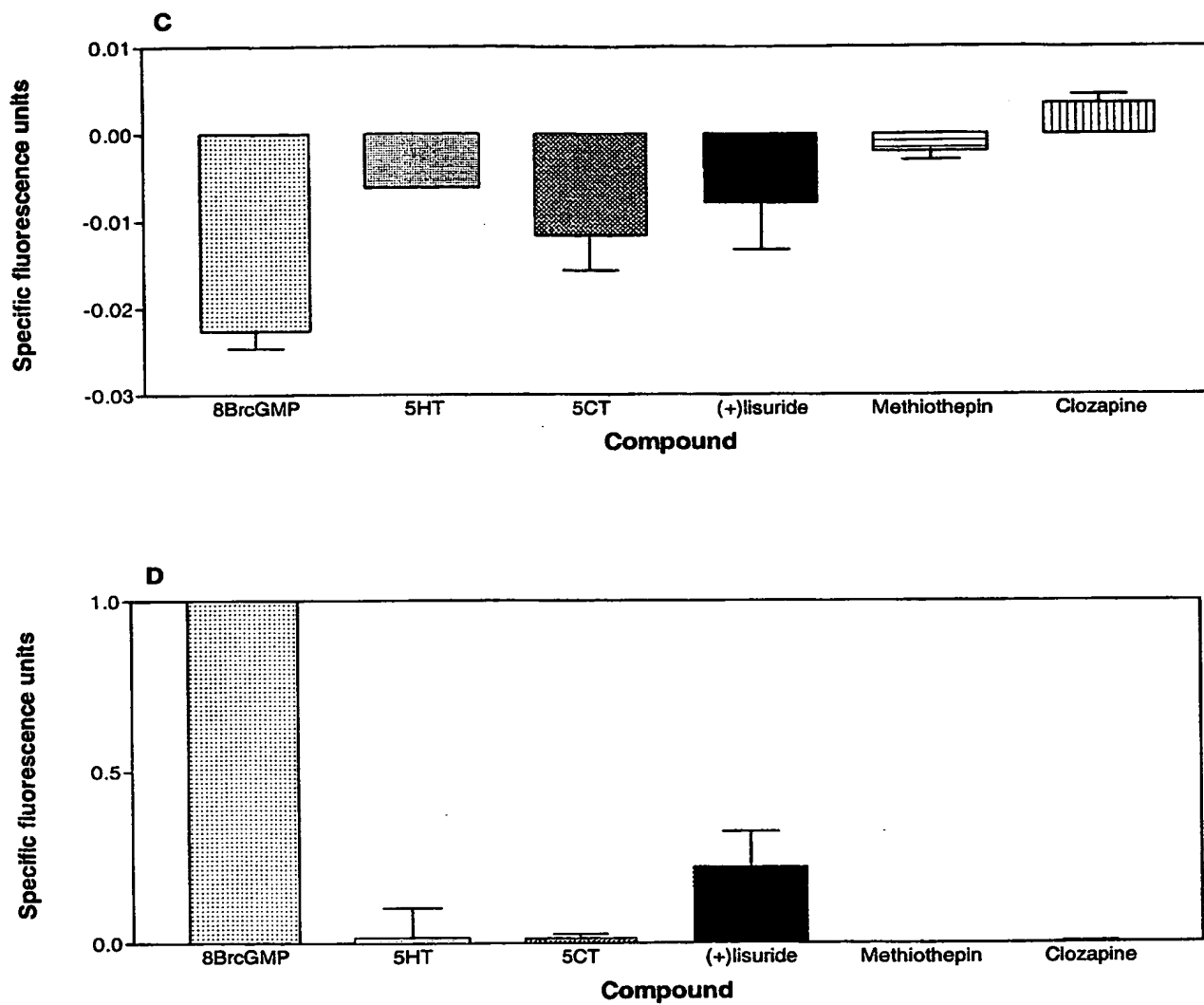


FIGURE 7

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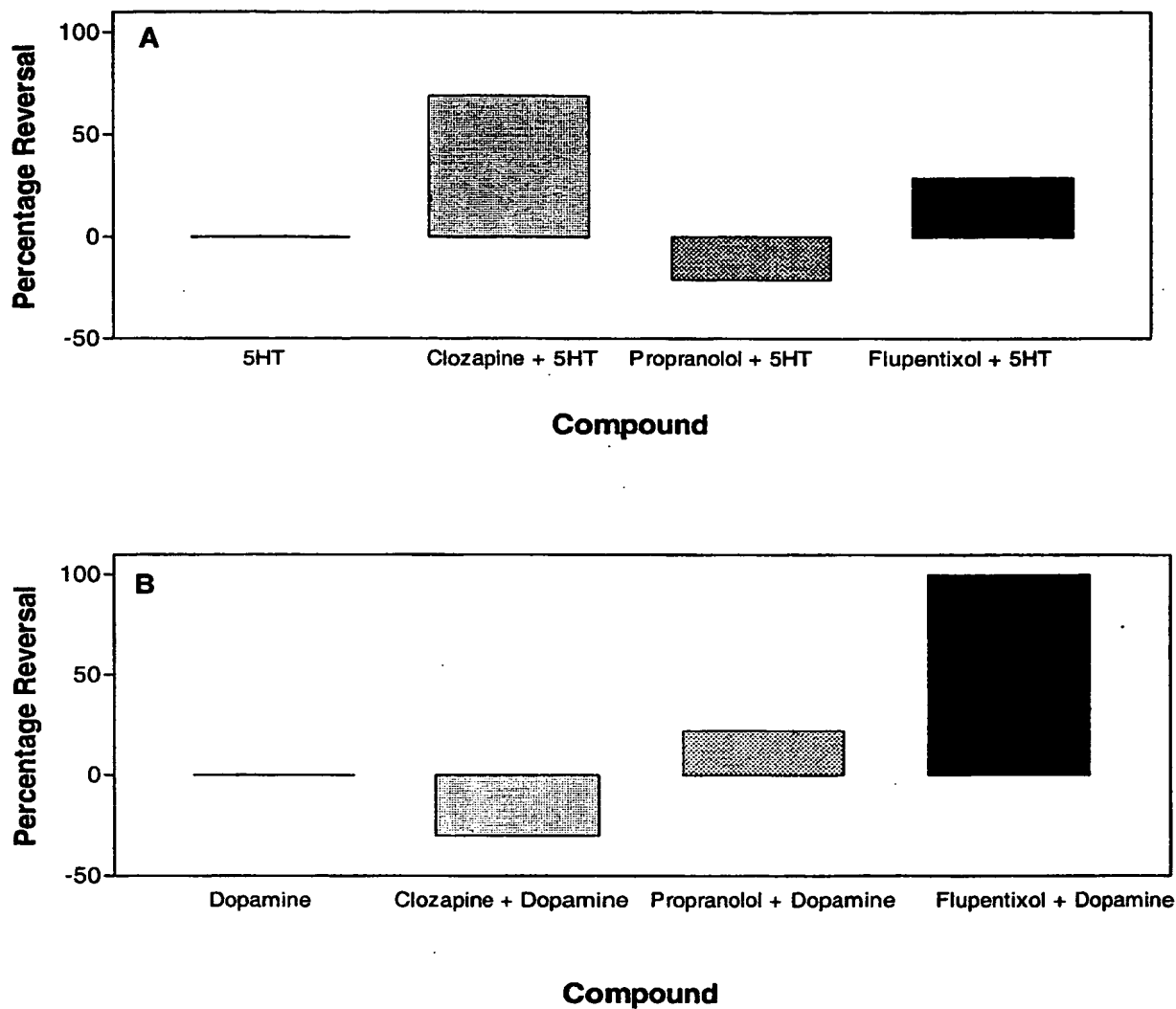


FIGURE 8



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/12, C07K 14/705, 14/72, G01N 33/50, C12N 5/10, G01N 33/68</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 98/58074</b> <b>(43) International Publication Date:</b> 23 December 1998 (23.12.98)
<b>(21) International Application Number:</b> PCT/CA98/00581 <b>(22) International Filing Date:</b> 12 June 1998 (12.06.98) <b>(30) Priority Data:</b> 08/874,663 13 June 1997 (13.06.97) US <b>(71) Applicant:</b> ALLELIX BIOPHARMACEUTICALS INC. [CA/CA]; 6850 Goreway Drive, Mississauga, Ontario L4V 1V7 (CA). <b>(72) Inventors:</b> BALLYK, Barbara, Ann; 2 Briarcroft Road, Toronto, Ontario M6S 1H4 (CA). ZASTAWNY, Roman; 21 Newstead Road, Etobicoke, Ontario M9P 3G2 (CA). LEE, David, K., H.; 5309 Forest Hill Drive, Mississauga, Ontario L5M 5B8 (CA). DEMCHYSHYN, Lidia; 4 Fernalroy Boulevard, Etobicoke, Ontario M8Z 3V6 (CA). CATALANO, Concettina; 119 Claremont Lane, Woodbridge, Ontario L4L 8Z8 (CA). <b>(74) Agents:</b> CHARI, Santosh, K. et al.; Orange & Associates, Toronto Dominion Bank Tower, Suite 3600, Toronto-Dominion Centre, P.O. Box 190, Toronto, Ontario M5K 1H6 (CA).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> <b>(88) Date of publication of the international search report:</b> 1 April 1999 (01.04.99)	
<b>(54) Title:</b> ASSAY METHODS AND COMPOSITIONS USEFUL FOR MEASURING RECEPTOR LIGAND BINDING <b>(57) Abstract</b> <p>This invention provides a system for screening chemical compounds to identify ligands for receptors including G-protein coupled receptors. The invention exploits cells in which the receptor is coupled through a second messenger system to an ion channel that is gated by cyclic nucleotide. Receptor stimulation causes the second messenger system to produce cyclic nucleotide, which results in ion influx through the channel. By measuring ion influx fluorescently, the invention provides a rapid and convenient means for identifying receptor ligands. By providing mixed cell cultures that include cells expressing different receptor types, and by loading into those cells different fluorescent reporters of ion influx, the invention further provides a multiplexed system that accelerates the ligand identification process. Cells useful in the process, and methods for exploiting them, are described.</p>		

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## INTERNATIONAL SEARCH REPORT

national Application No

PCT/CA 98/00581

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/705 C07K14/72 G01N33/50 C12N5/10  
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 13423 A (SALK INST BIOTECH IND) 8 July 1993	1,2,7-9, 18-22,25
Y	see abstract; claims 44,50-55; examples 1-3	3-6,23, 24
X	--- WO 94 20617 A (SALK INST BIOTECH IND ;ELLIOTT KATHRYN J (US); ELLIS STEVEN B (US)) 15 September 1994 see page 56, paragraph 3 - page 57, paragraph 1; claims 1,14-16,27	1,2,7-9, 18-22,25
X	--- WO 94 28131 A (CALIFORNIA INST OF TECHN) 8 December 1994 see page 14, line 17 - line 30 see page 20, paragraph 3 - page 21, paragraph 17	1,2,7, 18-22,25
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☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

28 October 1998

Date of mailing of the international search report

08.02.99

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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 98/00581

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VASUDEVAN S ET AL: "Muscarinic acetylcholine receptor produced in recombinant baculovirus infected Sf9 insect cells couples with endogenous G-proteins to activate ion channels." FEBS LETT, OCT 12 1992, 311 (1) P7-11, XP002082396 NETHERLANDS see the whole document ---	1,2,7, 18-22,25
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CA 98/00581

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This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
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1-12, 18-25

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-12 18-25

Method for identifying G protein coupled receptor ligands with a single type of cells expressing both an ion channel and a target receptor , corresponding method for screening chemicals , corresponding cells

2. Claims: 13-17 26-30

Method for screening screening chemicals to identify G protein coupled receptor ligands comprising a mixture of cells expressing an ion channel where both types of cells express different target receptor , corresponding mixture of cells

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 98/00581

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